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CD34 as a marker of angiogenesis in breast cancer: A study of 74 cases with brief review of literature

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ABSTRACT

CD34 was studied as a marker of angiogenesis in breast cancer. 74 cases of IDC were studied for this purpose. The mean microvessel density grade and the mean microvessel count per 400X field were determined for each case. Further, these 2 parameters were compared between node negative and node positive cases in order to determine whether increased angiogenesis would increase the chances of metastasis to lymph nodes. It was observed that cases with lymph node metastasis showed increased angiogenesis with a mean microvessel count and microvessel density grade of 45.6 and 2.91, respectively. In comparison, cases with absence of lymph node metastasis showed less angiogenesis and the mean microvessel count and microvessel density grade was 16.15 and 1.05 respectively. On statistical analysis, the difference between both the microvessel count and microvessel density grade for the two groups came out to be highly significant ($p<0.001$). Hence, increased angiogenesis was seen to lead to increased chances of metastasis to lymph nodes in our study.

Keywords: CD34, Angiogenesis, Breast, Breast cancer.

INTRODUCTION

There is considerable experimental evidence to prove that tumor growth is dependent on angiogenesis [1]. Thus after a new tumor has attained a small size of few millimeters in diameter (about 10^6 cells), further expansion of the tumor cell population requires the generation of new vessels. These new vessels also increase the opportunity of tumor cells to enter the blood circulation [2]. The first quantitative evidence that intensity of angiogenesis in a human tumor could predict the probability of metastasis was reported for cutaneous melanoma [3]. Later, many studies demonstrated the same in breast carcinoma [4, 5, 6, 7, 8].

Different studies have used different markers to highlight blood vessels in breast carcinoma in order to measure angiogenesis. The different antibodies that can be used are factor VIII, anti CD31 and anti CD34. Martin et al (1997) conducted a Meta-analysis and showed that anti-CD34 gave the most significant results of the three antibodies for

examining angiogenesis in cases of breast carcinoma [9].

CD34 has been widely used as a marker to assist in the identification and isolation of hematopoietic stem cells (HSCs) and progenitors in preparation for bone-marrow transplantation; more recently it has been employed as a marker to help identify other tissue-specific stem cells, including muscle satellite cells and epidermal precursors. Notably, however, the function of CD34 and its family members has not yet been definitively determined, although several roles have been ascribed to the proteins.

CD34 is a transmembrane, highly glycosylated protein expressed by hematopoietic stem/progenitor cells (HPSCs) [10], endothelial cells [11] and mesenchymal cells at several different sites, including breast [12]. It is thought to be involved in the modulation of cell adhesion and signal transduction [10].

Known tissue and cell types where CD34 is distributed include [13]

- Multipotent precursors: HSCs, multipotent haemopoeitic progenitors
- Mast cells
- Eosinophils
- Muscle satellite cells
- Hair follicle stem cells
- Vascular endothelia
- Fibrocytes
- Neurons +/-

REVIEW OF LITERATURE

Angiogenesis (or neovascularization) consists in the formation of new blood vessels from the endothelium of the existing vasculature. When a new tumor reaches the size of 1–2 mm, its ulterior growth requires the induction of new blood vessels, which may consequently lead to the development of metastases, via the penetration of malignant cells into the circulation. Vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor, and basic fibroblast growth factor, produced by tumor and stromal cells, are potent inducers of the angiogenic switch. However, angiogenesis is necessary but not sufficient to the development of metastases. Angiogenic activity is heterogeneous within a given tumor type. Concerning the relationship between angiogenesis and clinical outcome, breast cancer has been the most studied tumor. For >10 years, microvessel density (MVD), a surrogate marker of tumoral angiogenesis, has been proposed to identify patients at high risk of recurrence more precisely than classical indicators. The identification of such patients at an early stage of their disease would be of great interest, allowing for a more appropriate and effective treatment (by adjuvant chemotherapy or by specific antiangiogenic drugs) of patients at higher risk and possibly predicting the activity of these latter drugs.

Microvessel density assessment is the most commonly used technique to quantify Intratumoral angiogenesis in breast cancer. It was first developed by Weidner *et al.* in 1991 and uses panendothelial immunohistochemical staining of blood microvessels. CD34 is a known endothelial cell marker and is usually used for calculating microvessel density. The first step in Weidner's approach is the identification by light microscopy of the area of highest neovessel density (the so called "hot spot"), by scanning the whole tumoral section at low power. Then, individual microvessels are counted at a higher power (X200 field) in an adequate area (*e.g.*, 0.74 mm^2 per field using X20 objective lens and X10 ocular). Any stained endothelial cell or clusters separate from adjacent vessels are counted as a single microvessel, even in the absence of vessel lumen. Each single count is expressed as the highest number of microvessels identified at the hot spot (4). Some authors use Chalkley count or computerized image analysis systems, both aimed to minimize the subjectivity in the quantification of MVD. The Chalkley count consists of applying a 25-point eyepiece graticule on several hot spots (usually 3). The graticule is oriented to allow the maximum number of points to hit on or within the areas of stained microvessel profiles (Chalkley grid area: 0.196mm^2) [14].

Later in the year 1994, R. K. Vartanian and Weidner published a study titled" Correlation of intratumoral endothelial cell proliferation with microvessel density (tumor angiogenesis) and tumor cell proliferation in breast carcinoma" [15]. This study aimed to establish how intratumoral microvessel density correlates with tumor cell and intratumoral endothelial cell proliferation. The results of this study indicated no correlation between tumor cell proliferation (determined by

tumor cell Ki67 labelling index) and endothelial cell proliferation (determined by endothelial cell Ki67 labelling index). CD34 was also used to label microvessesls. Endothelial cell Ki67 labelling index did not correlate with intratumoral microvessel density or mitotic figure index, nor did intratumoral microvessel density correlate with tumor cell Ki67 labelling index or mitotic figure index. The authors concluded that intratumoral microvessel density, endothelial cell proliferation, and tumor cell proliferation may be regulated by separate mechanisms. Hence, it was becoming clear at this point that angiogenesis (as determined by microvessel density grade and microvessel count) may be a definite prognostic factor in women with breast cancer. However, the logical conclusion that increased tumor cell proliferation would directly lead to increased endothelial cell proliferation, higher microvessel counts and increased angiogenesis was disproven by this study.

In the year 1995, Siitonen et al conducted a study called "Comparison of different immunohistochemical methods in the assessment of angiogenesis: lack of prognostic value in a group of 77 selected node-negative breast carcinomas". They compared the different antibodies used to stain microvessels and the different quantitative methods used to measure angiogenesis. Anti-CD34 and anti-VWF showed better staining performances than anti-CD31, although the staining results with different antibodies were comparable. Two different methods of microvessel quantitation (the highest microvessel count and percentage microvessel area) were evaluated and also showed significant correlation. However, they found that neither highest microvessel counts nor microvessel area measurements quantitated with anti-CD34 or anti-VWF immunohistochemistry were able to discriminate between favourable and unfavourable outcome patients. They challenged the earlier view saying that further evidence was still needed on tumor angiogenesis immunohistochemistry before it could be adopted as a prognostic marker in routine, clinical practice [16].

In 1996, Heimann et al again proved the prognostic significance of angiogenesis in breast cancer by determining microvessel count (MVC) after highlighting microvessels by CD34. They correlated angiogenesis with clinical outcome in a series of patients with axillary lymph node-negative breast cancer who received no adjuvant therapy and who were followed for a long period of time. They found that angiogenesis as measured by MVC is a reliable independent prognostic marker of long-term survival in patients with node-negative breast cancer [17].

In 1998, concerns were raised by Hansen et al about the observer variability of methods for determining microvessel density. The microvessel endothelium was stained immunohistochemically by antibodies against CD34. The investigated methods included Chalkley counting, estimation of intratumoral microvessel density (MVD) by one hot-spot, MVD by the mean value of three hot-spots, and the highest value of MVD in three hot-spots. Each of forty tumors was measured with all methods, twice by the same observer and once by another observer. They found that the Chalkley and MVD methods had moderate reproducibility, and the Chalkley method had low variation due to observers alone. They concluded that the Chalkley method has less observer variation and may be superior from a methodologic point of view [18].

A new antibody was brought into focus in 1999 when Kumar et al assessed the microvessel density (IMD) in 106 breast carcinomas using a pan-endothelial marker, CD34, and a new mAb to CD105, which preferentially reacts with endothelial cell in angiogenic tissues. IMD values for CD105 expression showed a statistically significant correlation with overall (P = 0.0029) and disease-free survival (P = 0.0362). In contrast, blood vessel counts using a pan-endothelial marker CD34 did not correlate with overall or disease-free survival (P = 0.2912 and P = 0.3153, respectively). Multivariate analysis confirmed that IMD values using CD105 were an independent prognostic factor. The authors concluded that the ability to quantitatively distinguish between tumor neovascularization and preexisting vessels may be important in the assessment of tumor angiogenesis, but requires confirmation in a greater number of patients with a longer follow-up [19].

In 2000, Hansen et al conducted a study "Vascular grading of angiogenesis: prognostic significance in breast cancer" and again concluded that the angiogenesis determined by vascular grading (after staining for CD34) has independent prognostic value of clinical relevance for patients with breast cancer [20].

In 2004, Uzzan et al performed a meta-analysis of all 87 published studies linking intratumoral

microvessel density (MVD), reflecting angiogenesis, to relapse-free survival (RFS) and overall survival (OS). They found that MVD was a significant although weak prognostic factor in women with breast cancer [21].

In 2011, Mikalsen et al sought to decrease variability associated with different methods of microvessel quantification by introducing the method of automated vessel identification in CD34 immunohistochemical sections. They found this method to be highly reliable and recommended its use on colour photographs of staining for CD34 to quantify angiogenesis [22].

To summarize, many observational studies (either prospective or retrospective studies) have concluded that MVD is a prognostic factor in invasive breast cancer, but others reached the opposite conclusion. There have been concerns over the type of antibodies used and the method used for quantification. Therefore, although there is a consensus among most scholars that angiogenesis is an important factor determining outcome in women with breast cancers, the lack of standardization and high observer variability associated with different methods preclude its current clinical use as a prognostic factor.

MATERIALS AND METHODS

The present study was carried out on 74 patients of infiltrating ductal carcinoma, attending the OPD/IPD of Department of Surgery and on the histopathological specimens received in the Department of Pathology, J. N. Medical College, AMU, Aligarh. The study was conducted over a period of 2 years (from July 2012 to June 2014). A detailed clinical history and examination, along with routine investigations were carried out in each case.

Post-surgical specimens included mastectomies and a few biopsies. Gross examination of all the specimens was performed and sections were taken from the representative areas. They were processed by an automatic tissue processor (Histokinette). Blocks were prepared in paraffin wax with the help of Paraffin Embedding Station. Sections were cut at 4-5 µm thickness with the help of rotary microtome. All the cases were stained by Haematoxylin and Eosin and examined microscopically. Further, CD34 immunostaining was done on all the cases.

Immuno-histochemistry for CD34 was performed on paraffin embedded tissue sections using the kit, Thermo Scientific CD34 (Clone QBEnd/10). The antibody provided is prediluted and ready to use. Tissue was first microwaved in citrate buffer (at pH- 6.0, 95ºC, 10 min) for antigen retrieval. This was followed by peroxide block, protein block and incubation in primary antibody for 20 minutes at room temperature. Further incubation with HRP polymer and DAB was done. Finally, counterstaining with haematoxylin was done and dried, mounted slides were examined microscopically.

Staining for CD34 highlighted all the blood vessels. The method used for microvessel counting was modified from Weidner et al., (1991).

Tumors were frequently heterogeneous in their vessel density, however, the area of highest neovascularization was found by scanning the tumor section at low power (40X and 100X) and identifying the areas of invasive carcinoma with the highest number of discrete microvessels staining for CD34. After the area of highest neovascularization was identified, it was subjectively graded on a scale of +1 to +4. This was called the **microvessel density grade** .Then the individual microvessels were counted on a 400x microscopic field (i.e., 40X objective lens and 10X ocular lens). This was called the **microvessel count**. Any brown staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells and other connective tissue elements was considered a single, countable microvessel. Vessel lumina were not necessary for a structure to be defined as a microvessel (4).

Hence, a value for microvessel density grade and microvessel count was determined for each case. This data was analysed statistically to determine if there was a significant difference in the values for node positive and node negative patients. The test of significance used was student`s t test. A p value of less than 0.05 was considered as significant.

OBSERVATIONS

It was observed that cases with lymph node metastasis showed increased angiogenesis with higher values of microvessel count and microvessel density grade (Fig.1). In comparison, cases with

absence of lymph node metastasis showed less angiogenesis and low microvessel count and microvessel density grade (Fig. 2)

Microvessel density grade, microvessel count, tumor grade, tumor size and age of patients were compared between the node negative and node positive group. Difference in age and tumor grade was not found to be significant, while the rest of the parameters were significantly different in the 2 groups as described below:

Patients with breast carcinoma showing lymph node metastasis had a mean age of 48.68 while those without metastasis had the corresponding value of 48.08. No significant difference was found to be present between the two groups on statistical analysis ($p > 0.05$).

The mean tumor grade was 2.17 in patients with lymph node metastasis while it was 2 in patients without metastasis. The difference in the tumor grade between the two groups was not found to be statistically significant.

Microvessel density grade, microvessel count and tumor size were found to be significantly different between node positive and node negative patients.

As shown in table 1, the mean microvessel density grade was 2.91 in node positive cases with a median of 3. In comparison, the value in node negative patients was 1.05 with a median of 1. Statistical analysis revealed the difference between the 2 groups to be significant ($p <0.001$).

Also, in patients with metastasis to lymph nodes, the mean microvessel count was 45.6 per 400X field. In those without metastasis, the corresponding value was 16.15. Statistical analysis revealed the difference between these 2 groups also to be highly significant ($p <0.001$).

The mean tumor size was 6.54 cm. in cases with lymph node metastasis in comparison to 4.63 cm. in those without metastasis. Statistical analysis revealed the difference in tumor size between the two groups to be statistically significant ($p=0.001$).

Hence, it was observed that cases with lymph node metastasis had increased angiogenesis (seen in the form of high microvessel density grade and microvessel count), and higher mean tumor size.

Table 1: Clinico-histological characteristics & CD34 expression in 74 breast cancer cases, with or without lymph node metastasis.

CHARACTERISTIC	METASTASIS(–) (n=39)	METASTASIS(+) (n=35)	P VALUE
Microvessel density grade	1.05 ± 0.22 (1, 1-2)	2.91 ± 0.56 (3, 1-4)	<0.001**(significant)**
Microvessel count per 400x field	16.15 ± 3.29 (16, 10-22)	45.6 ± 9.35 (49, 25-65)	<0.001**(significant)**
Tumor grade	2 ± 0.73 (2,1-3)	2.17 ± 0.62 (2, 1-3)	0.146(notsignificant)
Tumor size (cm.)	4.63 ± 2.38 (4, 1-12)	6.54 ± 3.07 (6, 1-16.5)	0.003**(significant)**
Age (yrs)	48.08 ± 11.61 (50, 25-70)	48.68 ± 11.29 (48, 32-80)	0.42(not significant)

Values expressed as mean ± standard deviation (median, range).

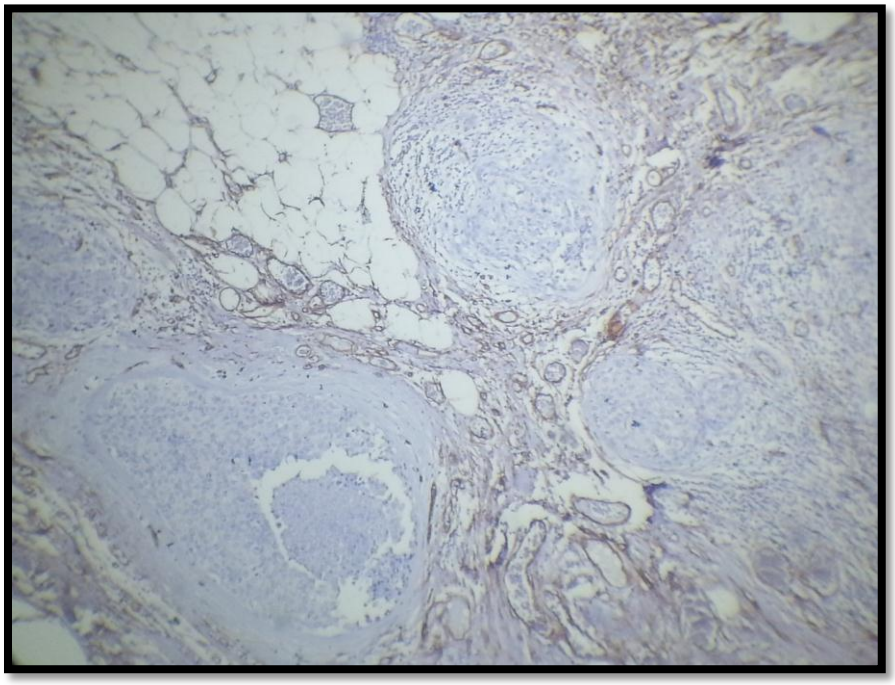

Fig. 1: A case of infiltrating ductal carcinoma with DCIS showing high microvessel density(CD34;40X)

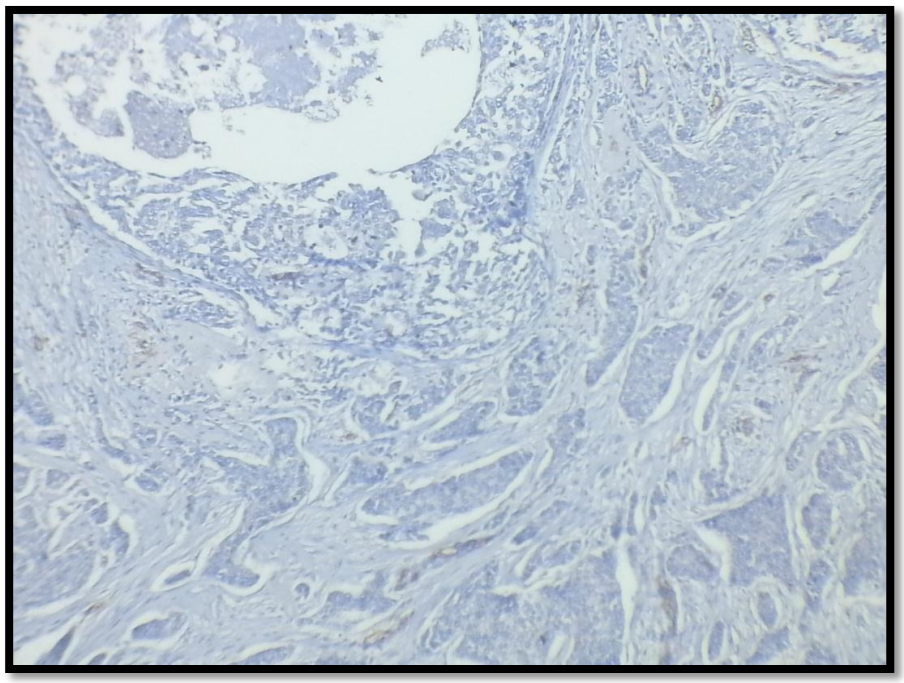

Fig. 2: A case of infiltrating ductal carcinoma with DCIS showing low microvessel density (CD34;40X)

DISCUSSION

Our findings were similar to those of Weidner et al (1991) who showed a significant correlation between the density of microvessels in histologic sections of invasive breast carcinoma and the occurrence of metastases. However, the marker used by Weidner to highlight the microvessels was factor VIII instead of CD34.

A significant correlation was also found between the size of IDC and lymph node metastasis. The mean tumor size was 6.54cm. for

cases with lymph node metastasis in comparison to 4.63cm. in those without metastasis. Statistical analysis revealed the difference in tumor size between the two groups to be statistically significant (p=0.001). It is already well known that the measured gross size represented by the largest diameter of a mammary carcinoma is one of the most significant prognostic variables. Numerous studies have shown that survival decreases with increasing tumor size and that there is a coincidental rise in the frequency of axillary zodal metastases [5,6,7,8]. Hence, our findings are consistent with different researchers earlier.

Although there has been much research in this field with newer antibodies and automated procedures introduced to detect angiogenesis in breast cancers, we sought to find out whether the oldest and simplest method introduced by Weidner et al still hold true. As suggested by our findings, it is obvious that this method showed a significant correlation between angiogenesis and lymph node metastasis. However, since this study was retrospective and did not involve follow up of the patients, we cannot comment on the effect of angiogenesis on patient outcome.

The role of CD34 as a marker of angiogenesis in breast cancer is still undecided. There are many studies which have proven that estimating angiogenesis via CD34 microvessel staining is a good method to predict patient outcome. However, there are also few with conflicting views on the topic. We are of the view that CD34 can definitely be used to measure angiogenesis in breast cancer and in turn can be utilized as a predictor of lymph node metastasis.

CONCLUSION

CD34 can be used for determining angiogenesis in breast cancer. Increased angiogenesis was seen to lead to increased chances of metastasis to lymph nodes in our study.

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